TERT promoter mutations and telomerase activity in urothelial carcinogenesis[Au:Edit OK?]

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Abstract

Telomerase activity imparts eukaryotic cells with unlimited proliferation capacity, one of the cancer hallmarks. Over 90% of human urothelial carcinoma of the bladder (UCB) are positive for telomerase activity. Telomerase activation can occur through several mechanisms. Mutations in the core promoter region of the human telomerase reverse transcriptase gene (*TERT*) cause telomerase reactivation in 60-80% of UCB, whereas the prevalence of these mutations is lower in urothelial cancers of other origins. *TERT* promoter mutations are the most frequent genetic alteration across all stages of UCB, indicating a strong selection pressure during neoplastic transformation.**[Au:We avoid formulations referring to the article authors ("we") in the abstract. I have edited the following text accordingly, OK?]** *TERT* **[Au:"promoter"?]** promoter mutations could arise during regeneration of normal urothelium**[Au:I tried to simplify here; is this what you meant?]** and due to consequential telomerase reactivation, might be the basis of UCB initiation, which represents a new model for the origination of urothelial carcinogenesis. In the future, *TERT* promoter mutations and telomerase activity might have diagnostic and therapeutic applications in UCB.

[Au:For your information, [H1] and [H3] denote the level of heading and will be removed during the production process. Where indicated, I have edited the headings to conform to the character limits of a Perspective article ([H1] 32 characters incl. spaces; [H3] 70 characters incl. spaces). Please adjust my edits of headings if you have better suggestions.]

[H1]Introduction

Telomerase is a ribonucleoprotein (RNA-containing protein) complex composed of the telomerase reverse transcriptase protein (TERT) and a telomerase RNA component (TERC), which assemble, in a cell cycle-dependent manner, with several accessory factors to form the active holoenzyme [1]. Telomerase is a telomere-specific enzyme that extends telomeres and its activity is required to overcome the end replication problem, counteracting the progressive loss of telomeric DNA at the tips of linear chromosomes during DNA replication. In the absence of telomerase activity, telomere shortening would eventually result in the loss of genetic material and genetic catastrophe owing to breakage-fusion-bridge (BFB) cycles. This cycle involves breakage of unstable chromosome structures (e.g. resulting from end-to-end-fusion of telomere-dysfunctional chromosomes) which may be broken during mitosis and initiate repeated rounds of fusion and breakage, leading to genome instability [2]. **[Au:Please reference. Edit for flow here OK? Please explain in the text what BFBs are. Thanks.]** Telomerase activity is maintained somatically in many animals, including the mouse, [3-7] but is down-regulated during human development and cellular differentiation through transcriptional repression of the catalytic subunit *TERT* gene [8-10].**[Au:Please clarify, is DNA expression repressed or the enzyme itself inhibited?]** In human adult non-germ tissues, *TERT* expression and telomerase activity is restricted to the stem or progenitor cell compartment of some tissues and can be activated in some cell types, such as lymphocytes, following proliferative stimulation [6,7,11-19].**[Au:With "emerges" did you mean "can be activated" as edited?]** In the absence of sufficient telomerase activity, telomeres shorten in proliferating cells during aging [20,21]. Short, dysfunctional telomeres lose capping function and activate DNA-damage responses. When DNA damage checkpoints are intact, telomere shortening limits the proliferative capacity of human cells and serves as a tumour suppressor mechanism by activating cell cycle arrest and senescence (Fig. 1).**[Au:Please can you add a brief mention of what activates the DNA damage response. Is it the BFBs?]** once the replication limit of ~50 cell divisions (known as the Hayflick limit) is reached [20-23].**[Au:As far as I am aware, strictly speaking, the term Hayflick limit is used to describe the number of times a cell can divide before excessive telomere shortening and senescence occur. How about reformulating to "A human somatic cell without telomerase activity usually reaches replicative senescence after ~50 cell divisions (the Hayflick limit)."?]** These observations are supported by *in vivo* data that show that telomere dysfunction, in combination with a defective p53 pathway, provokes BFB cycles and enhances genomic instability, eventually promoting tumorigenesis [24-27].

Telomerase activity is found in ~90% of human carcinomas (about 99% in UCB, see below), supporting the idea that activation of telomerase is an essential requirement for the immortalization (unlimited proliferation capacity) of human cells [28,29].**[Au:Although you mention it later on in the text, I think it is important to already mention here how frequent telomerase activity is in UCB, especially because you write how frequent ALT in UCB is, so that the reader has a clear comparison, OK?]** These observations led to the hypothesis that down-regulation of telomerase activity is a mechanism that protects from tumorigenesis. In line with this hypothesis, telomerase**[Au:add "activity" for specificity?]** activity is required for malignant conversion of primary human cells [30]. Tumours without telomerase maintain their telomere functionality via the alternative lengthening of telomeres mechanism (ALT) [31].**[Au:Please could you add a short explanation of what ALT involves?]** Stabilization of telomere length and functionality by ALT relies on homologous recombination of telomeric DNA between sister chromatids [32]. Recurrent mutations in the death-associated protein (DAXX) and the alpha-thalassemia X-linked protein (ATRX) have been correlated with ALT occurrence and potentially contribute to ALT maintenance [33,34]. Of note, there is evidence that telomerase activity supresses ALT, although the suppressive mechanism is yet to be identified [34-47]. In a comprehensive analysis of 6,110 human tumour specimens from various cancer types, ALT was observed in only ~4% of samples [48]. Importantly, the prevalence of ALT varied between cancers. Most astrocytomas and sarcomas rely on ALT [48-50], whereas telomerase activity is the primary mechanism for telomere maintenance in most other carcinomas [48,51].**[Au:Swapped sentence parts for flow, OK? I also deleted the specific examples, as they did not seem relevant.]** In urothelial carcinoma of the bladder (UCB), the prevalence of the ALT mechanism is low (~1%), indicating the importance of telomerase in this type of cancer [48].

Accumulating evidence shows that telomerase or its components have functions, independent of telomere lengthening, which affect many biological processes, including cell survival and apoptosis, DNA damage repair, mitochondrial function, cell adhesion and migration, and stem cell activity [34,52-61]. These alternative functions can be independent of the enzymatic activity of telomerase [62] and can involve activation of the WNT–ß-catenin signalling pathway by TERT [52,59].**[Au:We avoid the use of the ambiguous "may"; is "can" here OK or would "might" be better?]** However, the physiological relevance of the latter mechanism has been questioned [57,63].**[Au:Please excuse my ignorance but I am not sure how the previous thought (activation of the WNT–ß-catenin signalling pathway by TERT) and the processes in the following sentence (NFκB-dependent gene regulation 48 or ribosomal DNA[Au:OK?] transcription by RNA polymerase 1) are connected. Please could you elaborate in the text, so that also readers who are not familiar with these pathways can follow your discussion? Also, are you indicating that these pathways are relevant in tumorigenesis? If so, please mention specifically in the text, so that the reader is aware why these findings are**

mentioned in this Review.] Telomerase activity is required, but the presence of**[Au:OK?]** TERT alone is insufficient to modulate NFκB-dependent gene regulation [64] or ribosomal DNA**[Au:OK?]** transcription by RNA polymerase 1 [65].

Data are emerging that show that telomeres can sense cellular stress conditions and induce cellular senescence to protect from tumorigenesis [66-68].**[Au:Please reference.]** The cellular stress conditions can originate from chromosomal imbalances such as aneuploidy, oxidative damage, or hyperproliferation signals resulting from oncogene activation.**[Au:What specifically do you mean by "chromosomal imbalances". Please specify in the text. Please reference.]** [46,66,69,70]. Telomeres are able to form G-quadruplex (G4) structures, four-stranded nucleic acid structures that have been observed in guanine-rich DNA-regions [50,71-73].**[Au:OK? Please reference. Please explain what G-quadruplex structures are.]** G4 structures preferentially form during replication and transcription and could be detected at telomeres [74]. Current understanding is that the G-quadruplex structures impose a challenge to the replication machinery if not resolved properly [10,75,76]. Human telomeres contain 2,000–3,000 TTAGGG repetitions, which distinguishes telomeres from the rest of the genome and provides the basis for sensing genotoxic and oncogenic cell damage [77-82].**[Au:I think that we need some discussion of how the TTAGGG repetitions can serve as DNA damage sensors. I understand that this can be a large topic but the basic concept of how this mechanism works needs to be explained to reader. Please add. Thank you.]** There is evidence that the activity of specific helicases is required for the progression of the replication machinery to prevent replication fork stalling. Importantly, it was demonstrated that Pif1 helicase requires telomerase to resolve G4-structures at telomeres in *Saccharomyces cervisiae* [83]. Based on these observations, it was suggested that in the absence of telomerase, fork progression is impaired at telomeres due to replication fork stalling which finally can lead to DNA-double strand breaks [77]. Importantly, the oncogene-induced senescence (OIS) or aneuploidy-induced senescence (AIS) response is independent of telomere length and can be reduced by telomerase activity (Fig. 2) [46,68,84,85]. These data open a new perspective on the interplay of telomeres and telomerase in suppressing and promoting tumorigenesis [43,69].**[Au:By using "new perspective", it sounds a bit as if a previous view of the interplay of telomeres and telomerase in suppressing and promoting tumorigenesis exists. I am not really sure whether this is what you want to indicate and also what that previous theory was. Please clarify, and if you want to highlight that this is a change from previous paradigms then please summarize the previous paradigm of the interaction in one sentence, so that the reader can quickly grasp the difference. Thank you.]** This new idea indicates that in addition to the telomere shortening-induced tumor-protective function as described in Fig. 1, telomeres can act as a barrier to tumor progression under conditions such as oncogene-induced hyperproliferation, genotoxic oxidative damage or the presence of aneuploidy-inducing mutations (Fig. 2). Consequently, suppression of OIS or AIS by telomerase might enable telomerase-positive cells to acquire

tumour-initiating mutations that would normally trigger telomere-mediated senescence in telomerase-negative cells [43]. How the activity of telomerase can suppress the telomere-length-independent protective function of telomeres is still unclear.

In this Perspectives, we examine recent data showing a high prevalence of *TERT* promoter mutations in UCB, which occur early during tumorigenesis indicating that the biology of the urothelium provides a selection pressure for this change. **[Au:Please state specifically which data.]** We consider the implications of telomerase activity for UCB **[Au:Might it be better to specify "UCB"?]** cell survival in the context of telomere length-dependent and telomere length-independent roles of telomerase. We specifically also discuss the role of OIS and AIS abrogation in tumorigenesis.**[Au:OK?]** We present a new model for urothelial carcinogenesis that incorporates the role of telomerase activation and the potential of targeting telomerase as a therapeutic approach for UCB.

[H1]Telomerase in normal urothelium

Under physiological conditions, normal human urothelium is a long-lived, mitotically quiescent tissue with a low cell turnover rate but can develop a strong regenerative response and switch to a proliferative phenotype in response to damage [86-88]. In non-proliferating urothelium, telomerase is undetectable, but various reports have shown normal human urothelial (NHU) cells maintained in *vitro* as rapidly proliferating, finite (i.e. non-immortalized) cell lines to express transient, low-level telomerase protein expression and enzyme activity [11,13].**[Au:What are the *in vitro* conditions used to achieve proliferation? Please add, so that the reader can distinguish from the immortalized NHU cells mentioned below.]** Whether telomerase becomes activated in human bladder urothelium *in situ* in response to physiological stress or regenerative conditions is not known. The regulatory mechanisms underlying the repression of telomerase activity in quiescent human urothelium and its activation during proliferation of NHU cells remain to be revealed. Transient, low-level telomerase expression and activity might be involved in physiological regeneration of the tissue, but stable overexpression of *TERT* or clonal expansion of cells with a mutated *TERT* promoter is required for the immortalization of NHU cells [13,41,89]. Of note, immortalization of NHU cells is dependent on the fully functional telomerase holoenzyme, as a C-terminal tagged TERT-HA (TERT protein, fused to a short marker peptide sequence incorporating amino acids 98 to 106 of the human influenza hemagglutinin (HA)_protein) restored enzymatic activity but was incapable of inducing immortalization [90].**[Au:Please explain what TERT-HA is specifically and how this modification changes the enzyme. Does the TERT-HA actually elongate telomeres? How was enzymatic activity measured if not by telomere elongation?]** Telomerase-immortalized NHU cells remain genetically stable under standard

growth conditions *in vitro* [13,91]. However, when cultured under stress-imposing low-density conditions, genomic alterations that are similar to those seen in primary urothelial tumours**[Au:specifically urothelial tumours?]** can occur, such as loss of chromosomal region 2q [91]. In line with the extratelomeric functions of telomerase, forced expression of *TERT* in NHU cells results in loss of differentiation capacity[13,90] and gene expression changes including overexpression of the polycomb repressor complex (PRC1 and PRC4) components, BMI1 and SIRT1, and down-regulation of multiple PRC targets and genes associated with differentiation [90]).**[Au:Please give some examples of what specifically happens.]**

[H1]Telomerase and TERT promoter mutations

Telomerase activity, determined by the PCR-based telomerase repeated amplification protocol (TRAP) assay, has been found in 80–100% of UCB, but only in 2–5% of specimens from adjacent normal urothelium [92-94]. Similarly, telomerase activity was detectable in 62–100% of urinary samples from patients with UCB [92-94]. To date, only one study has implicated specific transcription factors in the regulation of telomerase activity in UCB cells [95]. Ectopic expression of the c-MYC oncoprotein activated *TERT* transcription and telomerase activity, whereas its counterpart MAD1 (mitosis arrest deficiency 1) had a repressive effect. Whether c-MYC and MAD1 affect telomerase activity in urothelial cells under physiological conditions is unknown.

Mutations in the promoter of the human *TERT* gene have been found in several cancer types, including UCB, and are the most common non-coding somatic mutations in cancer [58,96-101]. Of all UCB samples, 55–83% had *TERT* promoter mutations at one of the two positions responsible for maintaining telomerase activity in cancer cells [44,102-104].**[Au:Add ref#83 also here?]** Nine benign proliferative urothelial lesions (cystitis, nephrogenic adenoma, and inverted papilloma) only had the wild-type *TERT* promoter sequence [44].**[Au:OK? How specifically were these samples matched to the cancer samples?]** These observations indicate that urothelial cells with *TERT* promoter mutations arise de novo in a wild-type setting and that the mutation provides a selective advantage to the cell. The evidence further indicates that *TERT* promoter mutations have a tissue-specific role in urothelial tumorigenesis, being reported in different histological variants of primary bladder cancer, including small cell carcinoma. In the case of bladder adenocarcinoma, *TERT* promoter mutations were restricted to non-enteric type rather than enteric-type adenocarcinomas (the latter being typically of urachal or metastatic colorectal derivation) [39,44].**[Au:Please specify: which type are urachal or metastatic colorectal – nonenteric or enteric?]** Nevertheless, other tissue-regulatory factors might contribute to telomerase activity, as >90% of upper tract urothelial cancers

(UTUCs) encompassing renal pelvic carcinomas and ureteric cancers have telomerase activity [105,106], but only 43% and 19% of these tumours contain the two common *TERT* promoter mutations, respectively [107]. These findings suggest that alternative regulatory mechanisms contribute to establishing telomerase activity**[Au:OK?]** in UTUC compared with UCB, possibly owing to their different embryological derivations. Similarly, a particularly high prevalence of *TERT* promoter mutations (100%) has been reported for micropapillary urothelial cancer [108].

The two key positions for mutations in the *TERT* promoter are C228T and C250T (located at positions -124 and -146, respectively, relative to the ATG start codon) [100,109-111]. Mutations in the *TERT* coding region are rare (frequency < 0.5%) [112], but *TERT* promoter mutations occur in 60-80% of UCB samples and the C228T and C250T mutations together account for 99% of these mutations. Convincing experimental evidence exists that both these mutations create novel binding sites for the heterotetrameric GA-binding protein (GABP) transcription factor,**[Au:Are you referring to a specific subunit or the whole factor "GA-binding protein"?]** resulting in increased transcription of *TERT* and activation of telomerase [97,103,113].

*[H1]*TERT mutations as prognostic markers

Several studies have examined potential associations of *TERT* promoter mutations with UCB stage and grade. The studies report *TERT* promoter mutations in 59 to 79% (mean 70%) UCB irrespective of tumour stage or grade [44,100,102,114,115].**[Au:What specifically were these rates; could you provide a range of values?]** One team of researchers assessed disease-specific survival in correlation with *TERT* promoter mutations and *TERT* mRNA levels in two independent cohorts of chemotherapy-naïve patients (n = 35; n = 87), finding that the abundance of *TERT* mRNA, rather than promoter mutation itself, correlated strongly with reduced disease-specific survival [104].**[Au:Please can you mention the number of patients for context.]** They further showed that *TERT* promoter mutations correlated with *TERT* mRNA abundance, telomerase activity, and telomere length, compared with telomerase-positive cells without *TERT* promoter mutations. These observations might also explain the results obtained by another group, who analysed urine samples of 230 patients with non muscle invasive bladder cancer (NMIBC) and 25 samples of patients with muscle invasive bladder (MIBC) cancer. They found *TERT* promoter mutations to be most common mutation detected (52% across of all samples) and showed significant associations between *TERT* promoter mutation and progression of NMIBC to MIBC (9 of 110 patients who progressed had *TERT* promoter mutation versus 1 of 109 patients without). When NMIBC were stratified by risk, the presence of *TERT* promoter mutation was highly associated ($p<0.0001$) [40]. **[Au:Please can you mention the number of patients for context?]** Also of note, *TERT* promoter mutations

were found to be associated with distant metastases in UTUC [107], suggesting a selective advantage. In another study, sequencing of 76 urothelial carcinomas demonstrated *TERT* promoter mutations in both low-grade and high-grade UCB, as well as in flat and papillary lesions [116].**[Au:How does this finding relate to the results by Critelli? Did Kinde not see a difference in abundance depending on UCB grade? Why might this discrepancy exist? Please discuss in the text.]** The same mutations were also detectable in urine and were strongly associated with UCB recurrence, providing a potential prognostic**[Au:OK?]** urinary biomarker. The potential to use *TERT* promoter mutations as a urinary biomarker to detect UCB was explored in a prospective study using samples from 475 patients, which showed that *TERT* promoter mutations had the highest sensitivity (81.8%) but also the lowest specificity (83.5%) (among the markers tested: TERT, FGFR3, SALL3, ONECUT2, CCNA1, BCL2, EOMES, and VIM) to detect UCB [117].**[Au:Please mention which the other tested markers were for context.]**

[H1]TERT promoter mutations and cancer initiation[Au:Change to conform to character limit OK?]

Mutations that occur in adult stem cells are believed to have the largest effect on the mutational load of tissues, owing to their capacity to give rise to all descendant cells in a tissue [118,119]. Stem cell tracing experiments support this idea by showing that mutations that occur in stem cells are efficient cancer drivers, whereas the same mutations in differentiated cells fail to initiate cancer [120]. This view is supported by reports showing the accumulation of mutations in adult stem cells during life [37]. However, stem cells are rare and considered to divide infrequently, giving rise to a rapidly-expanding supra-basal compartment of highly proliferating transit-amplifying cells. In the past 5 years, an alternative cancer cell origin theory has emerged in favour of the transit-amplifying cell population. [121-123].**[Au:We avoid the use of "recently" as it can be interpreted differently by different readers; "In the past 5 years" instead OK? Also, please explain in the text what transit-amplifying cells are.]** This concept takes into account that mutation rates are increased in replicating cells [121] and that most heritable mutations occur in the transit-amplifying cells that constitute the majority of the stem or progenitor cell pool, rather than in the small number of rarely-dividing adult stem cells (Fig. 3).

The presence and nature of stem cells in human bladder urothelium is still under debate, but studies in animal models indicate that subpopulations of bladder urothelial cells (either P63-positive, SHH-positive, KRT5-positive, or KRT14-positive subpopulations)**[Au:For specificity: Do you indicate three subpopulations (P63+, SHH+, and KRT5+/KRT14+) or four subpopulations (P63+, SHH+, KRT5+, and KRT14+) here?]** confer self-renewal ability and are

considered to constitute the stem or progenitor cell population of the bladder epithelium [124-127]. In one study in mice, KRT14-positive cells, which are a subpopulation of KRT5-positive cells, gave rise to all cell types of the urothelium during injury-induced regeneration and were regarded as the cells of origin of urothelial cancer [128]. By contrast, studies with mouse and pig urothelial cells suggest that both the basal and intermediate cells can contribute to the regenerative potential of the bladder urothelium, indicating that a stringent requirement for a stem cell population does not apply to bladder urothelium [88,129]. These studies might be helpful in understanding the contribution of different cell types to bladder regeneration and cancer in different species but, unlike in human somatic tissues, telomerase is constitutively expressed in all mouse tissues [5,6].**[Au:Please reference again.]** Thus, irrespective of the stem cell debate, the common occurrence of *TERT* promoter mutations that result in constitutive telomerase activity demonstrates the critical importance of this tumour suppressor in human UCB.**[Au:Please can you reformulate here and specify what exactly the tumour suppressive block is in this context?]**

In a series of experiments performed on cell fractions enriched from isolated UCB and normal urothelium, one team of researchers located *TERT* promoter mutations (primarily the C228T mutation) in basal cells (defined as $CD44^+$ $CK5^+$ $CK20^-$) from UCB but not from normal urothelium (normal bladder basal cells; NBBC) [130].**[Au:Deletion of "lin-" OK, as seemed not required to define cell population?]** Furthermore, these *TERT* promoter mutations were enriched in basal compared with non-basal bladder cancer cells. Importantly, restoring the C228T mutation to the wild-type sequence abolished the tumour-forming ability of bladder cancer cells in mouse xenograft experiments. Conversely, basal urothelial cells isolated from **[Au:human?]** non-cancerous adjacent human bladder tissue expressed wild-type *TERT* and only developed xenograft tumours in nude mice when the C228T mutation was introduced at the genomic level, suggesting that *TERT* promoter mutation is a crucial event in the malignant transformation of human urothelium [130]. Pending formal demonstration, these data suggest that tumour-adjacent normal bladder urothelial cells have accumulated cancer-initiating genetic mutations that are not sufficient for malignant transformation by themselves but require activation of telomerase as the tumour-promoting step.**[Au:Please excuse if I missed it but does any evidence exist that the tumour-adjacent normal urothelial cells have other mutations already? The reader might wonder whether the TERT mutation could not also be sufficient for malignant transformation by itself. Please explain the dependency on other pre-existing mutations in the text. Thank you.]** Of note, it was demonstrated that telomerase is not an oncogene per se (Harley, 2002). One further observation from the above mentioned study was that human **[Au:human?]**NBBCs had no telomerase activity [130]. This feature differentiates NBBCs from intestinal stem cells and

haematopoietic stem cells, which have a constitutive telomerase activity.**[Au:Please add the values for comparison and reference.]** Consistent with the reports showing transient activation of telomerase in normal urothelial cells in culture [11,13],**[Au:Please reference again.]** it seems plausible that NBBCs might be able to up-regulate telomerase**[Au:add "expression"?]** **expression** transiently in response to regenerative signals.

The implications of these data in the context of cancer-initiating cells require further clarification.**[Au:We avoid open questions in our articles. Reformulated sentence OK?]** **[Au:"activity" or "expression"; please add.]** The presence or absence of telomerase activity in resting NBBCs has to be demonstrated unequivocally, but current data indicate that these cells are telomerase-negative [130] . In the absence of telomerase, harmful cancer-initiating mutations, such as oncogene activation (leading to OIS) or aneuploidy-inducing mutations (leading to AIS), would interfere with cell fitness and would induce premature senescence as a tumour suppressor mechanism (Fig. 2).**[Au:Are you referring to OIS and AIS here, which are mediated through the sensing ability of the telomeres specifically? I think the reader needs to be reminded of this connection here. In addition, please do ensure that you describe in more detail how this sensing works at the beginning of the article where I have asked for it. Thank you.]** We therefore suggest that, in UCB, the first tumour-initiating mutation is likely to be a telomerase re-activating mutation (for example, in the *TERT* promoter) in cells that have been activated for proliferation (Fig. 3).**[Au:I think to make this point, you need to ensure that you sufficiently discuss earlier in the text the "activated for proliferation" mutations that already exist in cells. Please add above where I have asked for it.]** Telomerase activity could then facilitate the survival of cells with secondary cancer-promoting mutations**[Au:Do you mean that these cancer-promoting mutations occur after the telomerase activating mutations? Are these then different from the "activated for proliferation" mutations mentioned in the preceding sentence? Please clarify.]** and prevent entry into cellular senescence. The fact that *TERT* promoter mutations occur preferentially or exclusively in tissues that have low or absent telomerase activity in their non-proliferating cells, such as bladder urothelium [98,99].**[Au:Please reference again.]** supports this hypothesis. In fact, high rates of *TERT* promoter mutations have been observed in liver cancer, and the liver is another mitotically quiescent highly regenerative tissue in which the identity of stem cells has not been clarified.**[Au:Edit for clarity OK? Is this also from ref#6, please reference.]** Human hepatocytes are telomerase-negative in the resting state but can up-regulate telomerase activity following stimulatory signals during regeneration [7].

In a study published in 2015, researchers introduced the common *TERT* promoter mutations into human embryonic stem cells (hESCs) and demonstrated that these mutations prevented down-regulation of the *TERT* promoter and telomerase activity during differentiation of these

mutant hESCs but not wild-type hESCs [131]. These data indicate that *TERT* promoter mutations that occur in stem or progenitor cells prevent differentiation and the shortening of telomeres.**[Au:You seem to only mention that telomerase activity is maintained but do not mention any readouts that relate to differentiation or specifically to telomere shortening. Were the researchers testing for these effects? Please provide further evidence to back up your statement.]** By contrast, the absence of *TERT* promoter mutations in cancers arising from highly proliferative tissues with a stem cell compartment [38,132]. **[Au:Please add examples and reference.]** might be explained by the inherently high telomerase activity of their stem cells [35]. This hypothesis assumes that, if the stem cells are the origin of *TERT* promoter mutations, the mutation rate of the *TERT* promoter would be expected to occur at a similar rate in all tissues.

[Au:I feel like this final summary of the proposed mechanisms would benefit immensely from an accompanying Figure. It seems that several different events occur in different sequence in different models. Please could you draft a schematic that summarizes this model?]

In summary, we hypothesize that *TERT* promoter mutations occur in telomerase-negative cells that are activated for proliferation "on demand". It is known that injury and physiological stimuli can induce telomerase activity in human tissues [133-136]. It was also shown that human *TERT* promoter is activated in response to liver injury in a mouse model carrying a human *TERT* promoter fragment in front of the lacZ reporter gene [7]. In the bladder urothelium, it seems plausible to assume that *TERT* promoter mutations occur in proliferating cells that have transiently activated telomerase in response to injury, which is in line with the alternative model of transit-amplifying cells as the cancer-initiating cells with the highest mutation rate (Fig. 3).**[Au:Please excuse if I missed it, but did you discuss evidence that telomerase is transiently activated in response to injury in bladder cells? If so please briefly reiterate here and reference.]** Most notably, no TERT promoter mutations could be found in an carcinogen-induced bladder cancer model that otherwise shows a mutational signature similar to human bladder cancer (e.g. Trp53) and recapitulates the molecular alterations of human muscle invasive bladder cancer [137]. The lack of *TERT* promoter mutations in this mouse model can be explained by the constitutive *TERT* gene expression (and constitutive telomerase activity) in mouse tissues as described above [3-7]. In light of the novel findings that telomerase can suppress senescence induction caused by genome instability (AIS) or oxidative stress (OIS),**[Au:Please reference. Is this OIS and AIS? Please excuse if I missed it, but I am not sure that you explain these specific findings in detail. Please expand the description of the studies that demonstrate these mechanisms.]** we extend this model to suggest that *TERT* promoter mutations are likely to be the first tumour-initiating mutations in tissues that lack telomerase activity in their non-proliferating cells. Interestingly, the lack of *TERT* promoter mutations in primary bladder adenocarcinoma suggests that this entity might

have a different origin of cancer [138]. This idea might also support the conclusion regarding the cell type of origin for UTUC,**[Au:OK?]** in which a low rate of *TERT* promoter mutations is observed despite activation of telomerase in the vast majority of UTUCs [105-107]. In urothelial tumours (of any origin) that lack *TERT* promoter mutations, whether telomerase reactivation is a late event caused by telomere dysfunction initiated genome instability or whether telomerase reactivation occurs early during cellular transformation, potentially promoting tumorigenesis by telomere-length-independent mechanisms, remains to be elucidated. Of note, telomerase reactivation is not the primary tumour-initiating event in these tumour types but might result from loss of negative regulatory factors that suppress *TERT* promoter activity **[Au:"activity"?]** in normal cells and/or from an aberrant**[Au:as in "increased"?]** increased expression of factors that positively regulate *TERT* gene expression [43].

*[H1]*Opportunities in diagnosis and therapy

The high frequency of specific *TERT* gene promoter mutations across all UCB grades and stages and its absence from adjacent histologically normal urothelium indicates an important function in both neoplastic transformation and maintenance of UCB [44,100,102,114,115].**[Au:Please reference again.]** This finding has led several groups to examine whether detection of *TERT* promoter mutations could be a urinary marker for bladder cancer detection [102]. A non-invasive liquid biopsy approach is highly attractive[139], as it might replace costly long-term cystoscopic surveillance for recurrence of low-grade non-invasive UCB and function as a screening tool for detecting UCB (particularly early detection of MIBC) in high-risk groups [117,140]. *TERT* promoter mutation detected in urine might not only be indicative of UCB but also of UTUC or renal cell carcinoma, although *TERT* promoter mutation rates in these tumour types are lower than in UCB [107,141].**[Au:Comparator added OK?]** One retrospective study has indicated that urinary *TERT* promoter mutation detection is not prognostic of UCB clinical outcome [102], but it might be a suitable marker for monitoring of disease recurrence [104].**[Au:Please move ref of study showing no prognostication to before the comma.]** However, large prospective studies are awaited to determine the value of any clinical test on the basis of *TERT* promoter mutation detection [142].

The presence of telomerase activity in most human cancers, along with mouse model data indicating its requirement for tumour progression, has inspired the development of telomerase inhibitors and their testing in preclinical studies [19,143-148]. UCB is a good candidate for telomerase-based therapies, as most UCBs are telomerase-positive. The first generation of inhibitors targeted telomerase activity itself, for example by stabilising the G-quadruplex [45]. The first of these inhibitors to enter phase II trials, imetelstat, showed promising clinical

activity against myeloproliferative neoplasms rather than solid tumours [149-151].**[Au:I am not sure that this study tested imetelstat also in solid tumours, which you currently seem to indicate. Please clarify. Was imetelstat tested in different tumour types in another study?]** This finding indicates the need to identify novel targets on the basis of the specific molecular understanding of telomeric and cancer-associated extra-telomeric functions of telomerase in UCB [34].

Alternative targeting of telomerase activity has been investigated with the nucleoside analogue 6-thio-2′-deoxyguanosine. This compound is a telomerase substrate precursor that becomes incorporated into telomeric DNA by telomerase during DNA replication, resulting in rapid telomere deprotection and apoptosis in telomerase-positive cancer cell lines originating from colon, (HCT116), from lung (A549) or liver (HCC15) while telomerase negative primary human fibroblasts (BJ) or telomerase-negative but ALT-positive immortal human fibroblast (AV13) were resistant to the treatment [152,153].**[Au:Which cells (tumour type) specifically? Also, please explain a bit more what telomere deprotection is. Thank you.]** The protein components of the telomere-protective shelterin complex, a complex of six protein components (telomeric repeat-binding factor 1 and 2 (TERF1 and TERF2, also known as TRF1 and TRF2), TRF2-interacting protein 1 (TERF2IP, also known as RAP1), TRF1-interacting nuclear factor 2 (TINF2, also known as TIN2), protection of telomeres protein 1 (POT1), and tripeptidyl-peptidase 1 (TPP1)) that specifically associate with telomeres, also offer alternative targets. These shelterin components protect telomere length and structure, partly by regulating telomerase activity and access to the telomeric DNA [154]. In cells with dysfunctional telomeres, chromosome ends are no longer protected efficiently by the shelterin components. These unprotected telomeres resemble chromosome breaks and activate the DNA-damage response [155-157]. Furthermore, they are prone to degradation by exonucleases and lose their capping function to protect from end-to-end fusions or loss of genetic material. This process of telomere-uncapping and de-protection can be induced by progressive telomere shortening in the absence of sufficient telomerase activity as well as by mutant or genetically-modified shelterin proteins, resulting in activation of p53-dependent senescence and/or apoptotic responses [158]. *PINX1* is of particular relevance in UCB, as it encodes a shelterin-recruited telomerase-regulatory protein and displays single-nucleotide polymorphisms that are associated with significantly reduced bladder cancer risk [159-161].**[Au:Journal style is to use "significant" only in a statistical context. Please add the P value or change to e.g. "substantially" OK?]** Hence, specific targeting of shelterin components or telomerase-associated proteins could be a useful strategy to modulate telomerase activity for UCB therapy.

In addition, evidence of non-canonical, extra-telomeric functions of telomerase and their utility as treatment targets is accumulating. As an example, eribulin, an anticancer drug that interferes with microtubule elongation, inhibits the RNA-dependent RNA-polymerase activity of telomerase [162]. Further understanding of the non-canonical roles of telomerase has potential for developing new cancer-specific therapeutics.

As the prevalence of the ALT mechanism is low or absent in UCB, telomerase targeting can be considered a promising first-line strategy. However, secondary activation of the ALT mechanism is a potential drawback of any telomerase-inhibiting therapy. Studies in a lymphoma-prone mouse model have shown that deletion of telomerase in tumours provoked activation of ALT in cancer cells as an adaptive response [163].**[Au:Which tumours specifically?]** Whether ALT activation is of concern in **[Au:"human"?]** human UCB is not yet clear, but research on the potential telomere-length-independent functions of telomerase gives reason to hope that telomerase inhibition might result in a telomere-length-independent senescence response that protects from tumorigenesis without activating the ALT mechanism. In the meantime, ALT itself remains a potential target.**[Au:I've changed "proleptic" to "potential", as I'm not sure all readers would understand. Is this the meaning you had in mind?]**

*[H1]*Conclusions

The high prevalence of telomerase activity in UCB and identification of two hot-spot mutations within the *TERT* promoter region as the most frequent genetic alteration across all disease stages indicate a very strong selection pressure for telomerase activation during neoplastic transformation. Three conclusions can be drawn from our current understanding of telomerase function in the initiation and maintenance of UCB and the prospects for developing cancer-specific therapies. First, *TERT* promoter mutations resulting in telomerase overactivity occur in telomerase-negative cells that reside in tissues without a well-defined telomerase-positive stem cell compartment. Notably, a potential transient function of telomerase in the regenerative response of urothelium remains an open question. Secondly, the observations that *TERT* promoter mutations are detectable in all stages of UCB, but not overtly normal urothelium, indicate that telomerase activation provides a survival advantage during the initial stages of urothelial tumorigenesis. This notion is in line with the non-canonical functions of telomerase in the abrogation of AIS and/or suppression of OIS. Thus, in the absence of telomerase, harmful cancer-initiating mutations, such as oncogene activation or aneuploidy-inducing mutations, would interfere with cell fitness and induce premature senescence as a tumour suppressor mechanism through sensing by telomeres.**[Au:through sensing by telomeres, or also alternative mechanism? Please clarify.]** By contrast, urothelial cells that acquire telomerase-activating *TERT* promoter mutations

obtain a survival advantage. This insight might lead to new strategies for cancer therapy. Finally, on the basis of the alternative cancer cell origin theory which suggests the transit-amplifying cells as the cell of origin of cancer, as described above [122,123],**[Au:Instead of mentioning the researcher names, please refer to (explain) this model as you have done in the main text above, so that the reader immediately knows what you are referring to.]** *TERT* mutations should occur in proliferating rather than in mitotically quiescent urothelial cells. These cells with *TERT* promoter mutations **[Au:"with *TERT* mutations"?]** are probably the cancer-initiating cells, which then accumulate additional oncogenic mutations during UCB progression. Consequently, detection of *TERT* promoter mutations in body fluids might be an early marker of UCB and potentially for early cancer therapy by targeting telomerase.

Competing interests statement

The authors declare no competing interests.

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Figure Legends

[Au:As you can see in the proofs, I have made some edits to the Figures for clarity and consistency with the legends, OK?]

Figure 1 | Classical concept of telomere and telomerase functions in tumour suppression and initiation. During the life of differentiated cells, telomere length shortens owing to suppression of telomerase expression**[Au:OK?]**. Short, dysfunctional telomeres resemble chromosome breaks, and activate various DNA-damage response mechanism [155-157]. After a maximum number of cell replications (Hayflick limit),**[Au:OK?]** critically short telomeres induce replicative senescence in cells with a functional DNA damage response (DDR), serving as a mechanism to protect from tumorigenesis.**[Au:Please add why cells with overly short telomeres are prone to tumorigenesis – why the DDR would be activated.]** In DDR-deficient cells, overly short telomeres can result in genetic instabilities by repeated breakage-fusion-bridges (BFB) cycles, that lead to cellular transformation. Transformed cells require telomerase activity to stabilize telomere functionality and avoid apoptosis, and for unlimited proliferation. Transformed cells in culture and human cancers usually reactivate telomerase or employ alternative lengthening of telomeres (ALT) as telomere maintenance mechanisms.

Figure 2 | Alternative concept of telomere and Telomerase functions in tumour suppression and initiation. a | New data indicate that tumour-initiating mutations (for example, an oncogenic mutation) occurring in a telomerase-negative somatic cell (T-) can cause telomere replication stress, accumulation of fragile telomeres, and eventual senescence independent of telomere length (oncogene-induced senescence (OIS) or aneuploidy-induced senescence (AIS). This senescence response prevents tumorigenesis. b | When the same mutation occurs in a telomerase-positive somatic cell (T+) with a telomerase-reactivating *TERT* promoter mutation, the mutated cell can continue to proliferate.**[Au:Please add why telomerase activation prevents senescence or apoptosis in a mutated cell.]** During continued proliferation, the cell accumulates additional mutations, eventually leading to transformation and cancer.

Figure 3 | The cell of origin of cancer in UCB. In tissues with a defined stem cell compartment, stem cells have constitutive telomerase activity, supporting the proliferation of stem and transit-amplifying cells. Telomerase activity is subsequently down-regulated in differentiated cells arising from the progenitor cells.**[Au:OK?]** In the mitotically quiescent bladder urothelium, including in basal cells, telomerase activity is undetectable. Whether telomerase is activated under physiological regenerative conditions in human urothelium *in*

vivo remains to be clarified. In urothelial carcinoma of the bladder (UCB), telomerase is activated through *TERT* promoter mutations (red flash icon), which probably occur in proliferating urothelial cells. The activation of telomerase endows continued proliferation of cells by preventing telomere shortening and counteracting cellular differentiation. Specifically, it was demonstrated that forced expression of *TERT* in normal urothelial cells results in loss of differentiation capacity[13,90], potentially by the down-regulation of components of polycomb response complex targets and genes associated with differentiation [90]. Cells with *TERT* mutations show sustained proliferation and can accumulate additional mutations (black flash icon) that promote malignant transformation.**[Au:For this Figure, please clarify the connection between the panel with the cells on the left and "Cellular differentiation" (how is differentiation blocked?), and with "Mutation load" (why does the mutation load decrease again?).]**

Author notes

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Fig 1

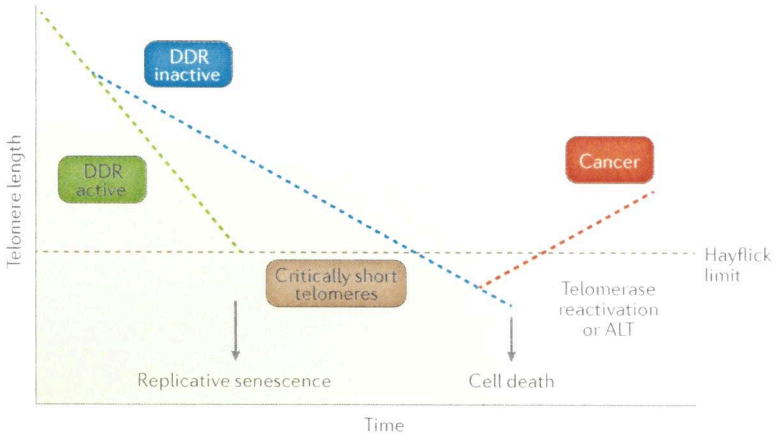

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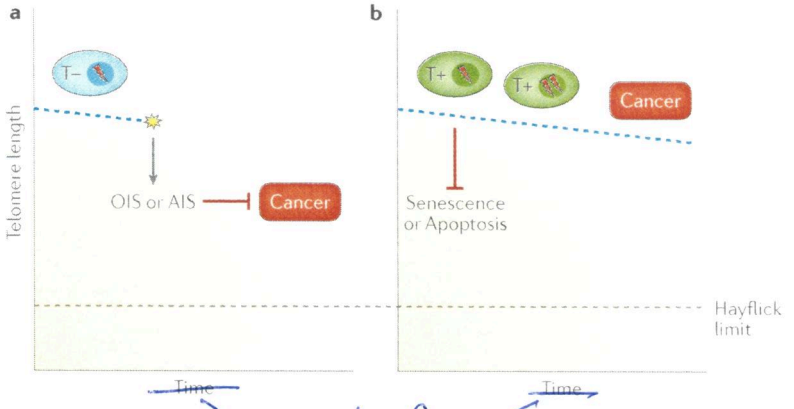
Fig 2



proliferation
(alternative: proliferation capacity)

Fig 3